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(54) Fusion Proteins for Prodrug Activation, the Preparation
and Use Thereof

(72) Seemann, Gerhard - Germany (Federal Republic of) ;
Bosslet, Klaus - Germany (Federal Republic of) ;
Czech, Joerg - Germany (Federal Republic of) ;
Kolar, Canek - Germany (Federal Republic of) ;
Hoffman, Dieter - Germany (Federal Republic of) ;
Sedlacek, Hans-Harald - Germany (Federal Republic of) ;

(73) Behringwerke Aktiengesellschaft - Germany (Federal
Republic of) ;

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BEHRINGWERKE AKTIENGESELLSCHAFT

HOE 91/B 004 2062047
Dr. Lp/Bi

Abstract of the Disclosure

Fusion proteins for prodrug activation, the preparation and use thereof

The invention relates to fusion proteins for prodrug activation of the general formula huTuMAB-L- β -Gluc, where huTuMAB is a humanized or human tumor-specific monoclonal antibody or a fragment thereof, L is linker, and β -Gluc comprises human β -glucuronidase. These fusion proteins are prepared by genetic manipulation. huTuMAB ensures the specific localization of tumors, L connects huTuMAB to β -Gluc in such a way that the specific properties of the two fusion partners are not impaired, and β -Gluc activates a suitable prodrug compound by elimination of glucuronic acid, where a virtually autologous system for use in humans is provided by the humanized or human fusion partners.

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BEHRINGWERKE AKTIENGESSELLSCHAFT HOC 91/B 004 - Ma 876
Dr. Lp/Bi

Description

- 5 Fusion proteins for prodrug activation, the preparation
and use thereof
-

10 The invention relates to fusion proteins for prodrug
activation of the general formula huTuMab-L- β -Gluc, where
huTuMab is a humanized or human tumor-specific monoclonal
antibody, a fragment or a derivative thereof, L is
linker, and β -Gluc comprises human β -glucuronidase. These
fusion proteins are prepared by genetic manipulation.
15 huTuMab ensures the specific localization of tumors, L
connects huTuMab to β -Gluc in such a way that the
specific properties of the two fusion partners are not
impaired, and β -Gluc activates a suitable prodrug com-
pound by elimination of glucuronic acid, where a
virtually autologous system for use in humans is provided
20 by the humanized or human fusion partners.

25 Combination of prodrug and tumor-specific antibody-enzyme
conjugates for use as therapeutic agents is described in
the specialist literature. This has entailed injection of
antibodies which are directed against particular tissue
and to which a prodrug-cleaving enzyme is covalently
bonded, into an animal which contains the transplanted
tissue, and subsequently administering a prodrug compound
which can be activated by the enzyme. The action of the
antibody-enzyme conjugate which is anchored in the tissue
30 converts the prodrug compound into the cytotoxin which
exerts a cytotoxic effect on the transplanted tissue.

35 WO 88/07378 describes a therapeutic system which contains
two components and is composed of an antibody-enzyme
component. The use of non-mammalian enzymes for the
preparation of the antibody-enzyme conjugate is described
in this case, and that of endogenous enzymes is ruled out

because of the non-specific release of active substance. Since the exogenous enzymes are recognized as foreign antigens by the body, the use thereof is associated with the disadvantage of an immune response to these non-endogenous substances, on the basis of which the enzyme immobilized on the antibody is inactivated and, possibly, the entire conjugate is eliminated. Furthermore, in this case p-bis-N-(2-chloroethyl)-amino-benzylglutamic acid and derivatives thereof are used as prodrug, the chemical half-life thereof being only 5.3 to 16.5 hours. The chemical instability of a prodrug compound is a disadvantage because of the side effects to be expected.

EP A2-0 302 473 likewise describes a therapeutic system containing two components, in which the antibody-enzyme conjugate which is localized on the tumor tissue cleaves a prodrug compound to give a cytotoxic active substance. The combined use, which is described herein *inter alia*, of etoposide 4'-phosphate and derivatives thereof as prodrug and antibody-immobilized alkaline phosphatases to liberate the etoposides is a disadvantage because of the presence of large amounts of endogenous alkaline phosphatases in serum. DE A1-38 26 562 describes how etoposide 4'-phosphates have already been used alone as therapeutic antitumor agent, with the phosphatases present in serum liberating the etoposide from the prodrug.

It has been found that huTUMAbs coupled via L to β -Gluc and prepared by genetic manipulation represent a particularly advantageous, because virtually autologous, system. It has additionally been found that the catalytic activity of β -Gluc in the fusion protein at pH 7.4 (i.e. physiological conditions) is significantly higher than that of the native enzyme when the fusion protein is bound to the antigen via the V region. Furthermore, a fusion protein with only one hinge region (see Fig. P and Example O) can be generated by genetic manipulation in high yield because most of the product which is formed

results as one band (in this case with molecular weight 125,000) and can easily be purified by affinity chromatography with anti-idiotypic MABs or anti-glucuronidase MABs.

5 It has furthermore been found that a chemical modification of the fusion proteins, in particular partial or complete oxidation of the carbohydrate structures, preferably with subsequent reductive
10 amination, results in an increased half-life. Enzymatic treatment of the fusion proteins according to the invention with alkaline phosphatase from, for example, bovine intestine or *E. coli* has in general not resulted in a significant increase in the half-life.

15 Consequently, the invention relates to fusion proteins of the formula

huTuMAB-L- β -Gluc (I)

where huTuMAB is a humanized or human tumor-specific monoclonal antibody or a fragment or a derivative thereof, and preferably comprises the MABs
20 described in EP-A1-0 388 914. The fusion proteins according to the invention particularly preferably contain the humanized MAB fragment with the V_L and V_H genes shown in Table 3.

25 L is a linker and preferably contains a hinge region of an immunoglobulin which is linked via a peptide sequence to the N-terminus of the mature enzyme.

β -Gluc is the complete amino-acid sequence of human β -glucuronidase or, in the relevant gene constructs, the complete cDNA (Oshima A. et al.,
30 Proc. Natl. Acad. Sci. USA 84, (1987) 685-689.

Furthermore preferred are constructs with a CH_1 exon and a hinge exon in the antibody part, and particularly preferred constructs are those in which these parts

derive from a human IgG3 C gene. Most preferred are constructs, as described in Example (I), where the corresponding light chain of the humanized TuMAb is co-expressed in order, in this way, to obtain an huTuMAb portion which is as similar as possible to the original TuMAb in the binding properties. Finally, the invention relates to processes for the preparation by genetic manipulation of the abovementioned fusion proteins, to the purification thereof and to the use thereof as pharmaceuticals. Fusion proteins as described can be used for prodrug activation in oncoses.

In another embodiment, the fusion proteins according to the invention are chemically modified in order to achieve an increased half-life and thus an improved localization of tumors. The fusion proteins are preferably treated with an oxidizing agent, for example periodate, which generally results in partial or complete cleavage of the carbohydrate rings and thus in an alteration in the carbohydrate structure. This alteration generally results in an increased half-life. It is furthermore advantageous to derivatize, in a second reaction step, existing aldehyde groups, for example by reductive amination. The partial or complete destruction of the aldehyde groups generally results in a reduction in possible side reactions with, for example, plasma proteins. Accordingly, it is advantageous for the fusion proteins according to the invention to be oxidized in a first reaction step, for example with periodate, and to be reductively aminated in a second reaction step, for example with ethanolamine and cyanoborohydride.

The following examples describe the synthesis by genetic manipulation of a particularly preferred fusion protein according to the invention, the derivatization thereof and the demonstration of the ability of the two fusion partners to function.

Example (A):

5 The starting material was the plasmid pGEM4-HUGP13 (Fig. A). pGEM4-HUGP13 contains a cDNA insert which contains the complete coding sequence for the human β -glucuronidase enzyme (Oshima et al. loc. cit.). All the techniques used were taken from Maniatis et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor USA (1989).

10 **Example (B):**

The plasmid pGEM4-HUGP13 was cut with the restriction endonucleases PstI and SalI, and the 342bp-long PstI/SalI fragment which harbors the NotI restriction cleavage site was isolated. The PstI/SalI fragment was cloned into the 15 PstI/SalI-cleaved vector pTZ (Fig. B.1) and the clone pTZ β Glc350 was isolated (Fig. B.2).

Example (C):

20 The plasmid clone pTZ β Glc350 was cleaved with PstI, and the double-stranded DNA fragment β Glc linker, which is composed of the oligonucleotides β Glc linker 1 and β Glc linker 2 (Tab. 1) and has cohesive PstI ends, was ligated into the opened PstI cleavage site.

BGlc Linker 1:

βGlc Linker 2:

5 The clone pTZ β Glc370 in which the β -glucuronidase fragment is extended at its 5' end by oligonucleotide I and which has lost the previously present PstI cleavage site but has acquired instead at its 5' end a new PstI cleavage site was isolated (Fig. C).

The plasmid clone pTZ β Glc370 was cleaved with PstI and ligated to the hinge-linker fragment which is composed of the hinge oligonucleotides 1 and 2b and which has two cohesive PstI ends (Tab. 2). This results in the PstI cleavage site at the 5' end of the β Glc370 fragment being destroyed. The plasmid clone pTZ β Glc420 in which the β Glc insert is extended at the 5' end by the hinge linker H was isolated (Fig. D).

Tab. 2

HincII 1 Glicc:

5' GAG CCC AAA TGT TGT GAC ACA CCT CCC CGG TGC CCA
3' CGG TGC CCA GTT GCA

HincII 2h Glicc:

5' ACT GGG CAC CCT GGG CAC GGG GGA GGT GTG TCA CAA
3' GAT TTG GGC TCT GCA

Example (E):

The plasmid pTZ β Glc420 was cleaved with PstI and SalI, and the 420bp insert was isolated. The plasmid
5 IgG3c F(ab'), 2H (EP-A2-0 352 761, Fig. 3, ibidem), which contains the CH₁ exon and two hinge exons of a human IgG3 C gene was completely cleaved with SalI and partially
10 cleaved with PstI. The isolated 420bp insert was ligated to this SalI/PstI (part.)-cleaved plasmid, and the plasmid clone which contains the CH₁ exon, a hinge exon and the β Glc420 fragment, that is to say carries the genetic information of two hinge exons between CH₁ exon and β -glucuronidase, was isolated (pUC CH₁ + H + β Glc420) (Fig. E).

Tab. 3

431/26_VH_hum

10	30	50
caactgcaggagagcggtccaggtcttgtgagacctagccagaccctgagcctgacct;		
GlnLeuGlnGlnSerGlyProGlyLeuValArgProSerGlnThrLeuSerLeuThrC;		
70	90	110
accgtgtctggcttcaccatcagcagtggttatagctggcactgggtgagacagccac;		
ThrValSerGlyPheThrIleSerSerGlyTyrSerTrpHisTrpValArgGlnProP;		
130	150	170
ggacgaggtcttgagtggttggatacatcacgtacagtggtatcactaactacaacc;		
GlyArgGlyLeuGluTrpIleGlyTyrIleGlnTyrSerGlyIleThrAsnTyrAsnP;		
190	210	230
tctctcaaaagtagagtgacaatgctggttagacaccagcaagaaccagttcagcctga;		
SerLeuLysSerArgValThrMetLeuValAspThrSerLysAsnGlnPheSerLeuA;		
250	270	290
ctcagcagcgtgacagccgcgacaccgcggtctattattgtgcaagagaagactatg;		
LeuSerSerValThrAlaAlaAspThrAlaValTyrTyrCysAlaArgGluAspTyrA;		
310	330	350
taccactggtactctcgatgtctggggtcaaggcagcctcgtcacagtcctcctca		
TyrHisTrpTyrPheAspValTrpGlyGlnGlySerLeuValThrValSerSer		

431/26_VX_hum

10	30	50
ggtgtccactccgacatccagatgacccagagcccaagcagcctgagcgccagcgtgg;		
GlyValHisSerAspIleGlnMetThrGlnSerProSerSerLeuSerAlaSerValG;		
70	90	110
gacagagtgaccatcacctgtagtaccagctcgagtgttaagttacatgcactggtacc;		
AspArgValThrIleThrCysSerThrSerSerSerValSerTyrMetHisTrpTyrG;		
130	150	170
cagaagccaggttaaggctccaagctgctgatctacagcacatccaacctggcttctg;		
GlnLysProGlyLysAlaProLysLeuLeuIleTyrSerThrSerAsnLeuAlaSerG;		
190	210	230
gtgccaagcagattcagcggtagcggtacggtaaccgacttcaccttcaccatcagca;		
ValProSerArgPheSerGlySerGlySerGlyThrAspPheThrPheThrIleSerS;		
250	270	290
ctccagccagaggacatcgccacctactactgccatcagtgagtagttatccacgt;		
LeuGlnProGluAspIleAlaThrTyrTyrCysHisGlnTrpSerSerTyrProThrP;		
310	330	
ggccaagggaccaaggtggaatcaaacgt		
GlyGlnGlyThrLysValGluIleLysArg		

Example (F):

5 The plasmid pGEM4-HUGP13 β Glc was cleaved with SalI, and the 1750bp SalI fragment from the β -glucuronidase cDNA was isolated. The isolated 1750bp SalI fragment was ligated to the SalI-cleaved plasmid pUC CH₁ + H + β Glc420. The plasmid clone pUC CH₁ + H + hu β Glc which contains a fusion gene composed of a CH₁ exon, a hinge exon and a fusion exon between a hinge exon and the human β -glucuronidase cDNA was isolated (Fig. F).

10 Example (G):

The expression vector pABstop (Fig. I) was cleaved with HindIII and SalI. The plasmid pUC CH₁ + H + hu β Glc was cleaved completely with HindIII and partially with SalI, and the CH₁ + H + hu β Glc insert was isolated. The CH₁ + H + hu β Glc insert was ligated to the HindIII/SalI-cleaved pABstop, and the clone pABstop CH₁ + H + hu β Glc was isolated (Fig. G).

Example (H):

20 The pABstop vector pABstop BW 431/26 hum V_H, which contains the humanized version of the V_H gene of the anti-CEA MAb BW 431/26 (Bosslet K. et al., Eur. J. Nucl. Med. 14, (1988) 523-528) (see Tab. 3 for the sequences of the humanized V_H and V_L gene), was cleaved with HindIII and BamHI, and the insert which contains the signal exon and the V_H exon was isolated. The plasmid clone pABstop CH₁ + H + hu β Glc was cleaved with HindIII and ligated to the HindIII/BamHI 431/26 hum V_H fragment. After ligation at room temperature for 2 h, the ligation was stopped by incubation at 70°C for 10', and the ends which were still free were filled in with Klenow polymerase and dNTPs. Further ligation was then carried out overnight. After transformation, the clone pABstop 431/26 hum V_H hu β Glc1H which contains an immunoglobulin P(ab')₂ gene with a hinge exon, which is fused to the coding region of human

β -glucuronidase, was isolated with the aid of restriction mapping and nucleic acid sequence analysis (Fig. H).

Example (I):

5 The clone pABStop 431/26 hum V_H hu β Glc1H was transfected together with a plasmid clone which carries the light chain of humanized BW 431/26 (Fig. I) and two plasmids which carry a neomycin- (Fig. K) and a methotrexate-resistance gene (Fig. L) into BHK cells. A fusion protein
10 MAb BW 431/26hum and the enzymatic activity of human β -glucuronidase was expressed.

Example (J):

Demonstration of the antigen-binding properties and of the enzymatic activity of the 431/26 hum V_Hhu β Glc 1H
15 fusion protein

The ability of the 431/26 hum V_Hhu β Glc 1H fusion protein to bind specifically to the epitope defined by 431/26 on CEA (carcinoembryonic antigen) and, at the same time, to exert the enzymatic activity of human β -glucuronidase was
20 determined in a specificity/enzyme activity assay. This assay is carried out as described below:

- Polystyrene (96-well) microtiter plates (U shape, Type B, supplied by Nunc, Order No. 4-60445) are incubated with purified CEA (1-5 μ g of CEA/ml, 75 μ l of this per well) or with GIT mucin (same amount as CEA) at R.T. overnight.
25
- The non-adsorbed antigen is removed by aspiration and washed 3 x with 0.05 M tris/citrate buffer, pH 7.4.
- 30 - The microtiter plates are left to stand at R.T. with the opening facing downwards on cellulose overnight.
- The microtiter plates are incubated with 250 μ l of 1% strength casein solution in PBS, pH 7.2, per well

(blocking solution) at 20°C for 30 minutes.

- During the blocking, the substrate is made up. The amount of substrate depends on the number of supernatants to be assayed. The substrate is made up fresh for each assay.
- 5 - Substrate: 4-methylumbelliferyl β -D-glucuronide (Order No.: M-9130 from Sigma), 2.5 mM in 200 mM sodium acetate buffer, pH 5.0, with 0.01% BSA.
- 10 - The blocking solution is removed by aspiration, and in each case 50 μ l of BHK cell supernatant which contains the fusion protein are loaded onto the microtiter plate coated with CEA or GIT mucin (that is to say the sample volume required is at least 120 μ l).
- 15 - Incubation at R.T. is then carried out for 30 minutes.
- The plates are washed 3 x with ELISA washing buffer (Behring, OSEW 96).
- 20 - The substrate is loaded in (50 μ l/well) and incubated at 37°C for 2 hours. The plate is covered because of the possibility of evaporation.
- After 2 hours, 150 μ l of stop solution are pipetted into each well (stop solution = 0.2 M glycine + 0.2% SDS, pH 11.7).
- 25 - Evaluation can now be carried out under a UV lamp (excitation energy 380 nm) or in a fluorescence measuring instrument (Fluoroscanner II, ICN Biomedicals, Cat. No.: 78-611-00).

30 It was possible to show using this specificity/enzyme activity assay that fluorescent 4-methylumbelliferol was detectable in the wells coated with CEA when the enzyme activity was determined at pH 5, the catalytic optimum (Tab. 4).

Table 4:

Dilution out of fusion protein cell culture supernatant (B73/2) on CEA and GIT mucin

5	Dilution steps	Substrate in various solutions		
		0.2 M sodium acetate buffer + 0.01% BSA, pH 5, on CEA	PBS, pH 7.2, on CEA	PBS, pH 7.2 on GIT mucin
10	Concen- trated	9118	2725	115.7
	1:2	7678	2141	93.37
	1:4	4662	1195	73.39
	1:8	2927	618.5	60.68
15	1:16	1657	332.1	53.69
	1:32	853	168.2	40.44
	1:64	425	99.26	48.21
	1:128	192.5	57.89	47.48

20 Determination of the conversion rate at pH 7.2 showed that at this physiological pH that of the fusion protein was still ~ 25% of the conversion rate at pH 5. No significant methylumbelliferol liberation was measurable on the negative control plates coated with GIT mucin and measured at pH 5. This finding shows that the humanized

25 V region of the 431/26hum V_HμβGlc 1H fusion protein has retained its epitope specificity, and the β-glucuronidase portion of the fusion protein is able, like the native human enzyme, to cleave the β-glucuronide of 4-methylumbelliferol.

30 Example (K):

Demonstration of the functional identity of the V region of the 431/26hum V_HμβGlc 1H fusion protein with that of the humanized MAb BW 431/26 and that of the murine MAb BW 431/26

It was shown in Example (J) that the 431/26hum V_hμpGlc 1H fusion protein has a certain CEA-binding potential and β-glucuronidase activity. The antigen-specific competitive assay described hereinafter provides information on the identity of the CEA epitopes which are recognized by the competing molecules, and on the strength of the epitope/fusion protein and epitope/antibody interactions.

This assay is carried out as described below:

- 10 - Polystyrene 96-well microtiter plates (U shape, Type B, supplied by Nunc, Order No. 4-60445) are incubated with purified CEA (1-5 μg of CEA/ml, 75 μl of this per well) or with GIT mucin (same amount as CEA) at R.T. overnight.
- 15 - The non-adsorbed antigen is removed by aspiration and washed 3 x with 0.05 M tris/citrate buffer, pH 7.4.
- The microtiter plates are left to stand at R.T. with the opening facing downwards on cellulose overnight.
- 20 - The microtiter plates are incubated with 250 μl of 1% strength casein solution in PBS, pH 7.2, per well (blocking solution) at R.T. for 30 minutes.
- 50 μl of the MAb BW 431/26 in a concentration of 5 ng/ml are mixed with 50 μl of 10-fold concentrated supernatant of the humanized MAb BW 431/26 or of the fusion protein, as well as serial 2 x dilutions.
- 25 - 50 μl aliquots of these mixtures are pipetted into the wells of microtiter plates coated with CEA or GIT mucin.
- The microtiter plates are incubated at R.T. for 30 minutes.
- 30 - The plates are then washed 3 x with ELISA washing buffer (supplied by Behringwerke AG, Order No. OSEW 96, 250 μl).
- Then 50 μl of a 1:250-diluted goat anti-mouse Ig antibody which is coupled to alkaline phosphatase (Southern Biotechnology Associates, Order No.: 1010-04) are added.
- 35

- After incubation at R.T. for 30 minutes and washing 3 times, the substrate reaction is carried out as follows:
- 5 - Add 30 μ l of 0.1 mM NADP per well (dissolve 7.65 mg in 100 ml of 20 mM tris; 0.1 mM MgSO_4 , pH 9.5); the solution can be stored at -20°C for several months.
- Incubate at R.T. for 30 minutes.
- Make up the enhancer system during the incubation with NADP: (5 ml per plate)
- 10 2 parts of INT (dissolve 2.5 mg/ml in 30% strength ethanol in an ultrasonic bath; always make up fresh)
 + 1 part of PBS, pH 7.2
 + 1 part of diaphorase (1 mg/ml PBS, pH 7.2)
 + 1 part of ADH (0.5 mg/ml PBS, pH 7.2)
- 15 - add 50 μ l of the enhancer system
- when the extent of reaction is as required, stop the reaction with 0.1 N H_2SO_4 , 100 μ l per well
- measure at 492 nm in a TITERTEK[®] MULTISCAN (blank = 50 μ l of NADP + 50 μ l, of enhancer solution +
20 100 μ l of 0.1 N H_2SO_4)

NADP - supplied by Sigma, Order No. N-0505

INT - supplied by Sigma, Order No. I-8377

ADH - supplied by Sigma, Order No. A-3263

Diaphorase - supplied by Sigma, Order No. D-2381

- 25 Reduction of the extinction in this antigen-specific competitive assay means that there is competition between the molecules competing with one another for epitopes which are the same or lying very close together spatially.

- 30 The inhibition data which are obtained show that both the fusion protein 431/26hum $\text{V}_\text{H}\mu\text{gGlc}$ 1H and the humanized MAb 431/26 block binding of the murine MAb BW 431/26 to its CEA epitope. 50% inhibition is reached at a 200 molar excess of the relevant competitors. The conclusion from
35 this is that the avidity of the fusion protein for the CEA epitope is comparable with that of the humanized

MAb 431/26. Furthermore, the fusion protein and the humanised MAb bind to the same epitope or to an epitope which lies spatially very near to that defined by the murine MAb BW 431/26 on CEA.

5 **Example (L):**

Demonstration of the tissue specificity of the 431/26hum V_HhuβGlc 1H fusion protein

10 Example (J) showed, inter alia, that the 431/26hum V_HhuβGlc 1H fusion protein is able to bind to purified CEA.

15 Example (K) showed that the V region of the fusion protein is able to compete with the V region of murine BW 431/26 for the same, or a very close, epitope. The indirect immunohistochemical assay which is specific for β-glucuronidase and is described hereinafter can be used to determine the microspecificity of the fusion protein on cryopreserved tissues.

The assay is described below:

- 20 - 6 μm-thick frozen sections are placed on slides and dried in air for at least 30 minutes.
- The slides are subsequently fixed in acetone at -20°C for 10 seconds.
- The slides are washed in tris/NaCl washing buffer, pH 7.4, with 0.1% BSA for 5 minutes.
- 25 - 20-100 μl of fusion protein-containing BHK cell supernatant (concentrated or diluted in tris/BSA, pH 7.4) is applied to each section and incubated in a humidity chamber at R.T. for 30 minutes.
- The slides are washed in tris/NaCl washing buffer, pH 7.4, with 0.1% BSA for 5 minutes.
- 30 - 50 μl of hybridoma supernatant of the murine anti-β-glucuronidase MAb BW 2118/157 are added to each section, and the slides are incubated in a humidity

chamber at R.T. for 30 minutes.

- The slides are then washed in tris/NaCl washing buffer, pH 7.4, with 0.1% BSA for 5 minutes.
- 5 - 20-100 μ l of bridge Ab (rabbit-antimouse IgG diluted 1:100 in human serum, pH 7.4) are applied to each section and incubated in a humidity chamber at R.T. for 30 minutes.
- The slides are then washed in tris/NaCl washing buffer, pH 7.4, with 0.1% BSA for 5 minutes.
- 10 - Subsequently 20-100 μ l of APAAP complex (mouse anti-AP diluted 1:100 in tris/BSA, pH 7.4) are applied to each section and incubated in a humidity chamber at R.T. for 30 minutes.
- The slides are then washed in tris-NaCl washing buffer, pH 7.4, with 0.1% BSA for 5 minutes.
- 15 - The substrate for alkaline phosphatase is made up as follows (100 ml of substrate solution sufficient for one glass cuvette):
Solution 1: 3.7 g of NaCl
20 2.7 g of tris base (dissolve in 75 ml of distilled water)
+ 26.8 ml of propanediol buffer, pH 9.75, adjust with HCl
25 + 42.9 mg of levamisole = clear, colorless solution
Solution 2: Dissolve 21.4 mg of sodium nitrite in 535 μ l of distilled water
= clear, colorless solution
30 Solution 3: Dissolve 53.5 mg of naphthol AS BI phosphate in 642 μ l of dimethylformamide (DMF)
= clear, yellowish solution
- Add 368 μ l of 5% strength new fuchsin solution to
35 solution 2 (sodium nitrite) and leave to react for 1 minute (stopclock) to give a clear, brown solution
- Add solution 2 (sodium nitrite with new fuchsin) and solution 3 (naphthol AS BI phosphate) to solution 1 (tris/NaCl/propanediol buffer) = clear,

yellowish solution

- adjust to pH 8.8 with HCl - cloudy, yellowish solution
- filter solution and place on the slide and leave to react on a shaker for 15 minutes - solution becomes cloudy.
- wash slide in tris/NaCl buffer, pH 7.4, for 10 minutes
- wash slide in distilled water for 10 minutes
- after drying in air for 2 hours, the slides are sealed in Kaiser's glycerol/gelatin at 56°C.

Specific binding of the fusion protein was demonstrated under the light microscope by the red coloration of the epitope-positive tissue sections. Comparative investigations with the murine MAb BW 431/26, which was detected by the indirect APAAP technique (Cordell et al., J. Histochem. Cytochem. 32, 219, 1984), revealed that the tissue specificity of the fusion protein agreed exactly with that of the murine MAb BW 431/26, i.e. that there is identity both of the reaction type in the individual specimen and of the number of positive and negative findings from a large number of different carcinomas and normal tissue.

Example (M):

Purification of the 431/26hum V_Hhu β Glc 1H fusion protein

Murine and humanized MAbs can be purified by immunoaffinity chromatography methods which are selective for the Fc part of these molecules. Since there is no Fc part in the 431/26hum V_Hhu β Glc 1H fusion protein, it was necessary to develop an alternative highly selective immunoaffinity chromatography method. Besides the selectivity of this method to be developed, it is necessary for the isolation conditions to be very mild in order not to damage the β -glucuronidase, which is very labile in the acidic and in the alkaline range.

The principle of the method comprises purification of the fusion protein from supernatants from transfected BHK cells using an anti-idiotypic MAb directed against the humanized V region. The preparation of such MAbs is known from the literature (Walter et al., Behring Inst. Mitt., 82, 182-192, 1988). This anti-idiotypic MAb can be both murine and humanized. The MAb is preferably immobilized on a solid phase so that its V region has not been damaged. Examples of this are known from the literature (Fleminger et al., Applied Biochem. Biotechnol., 23, 123-137, 1990; Horsfall et al., JIM 104, 43-49, 1987).

The anti-idiotypic MAb thus immobilized on the solid phase by known methods binds very efficiently the fusion protein to be purified from transfected BHK cells, for example at pH 7, but has the surprising property that it no longer binds the fusion protein when the pH is lowered by only 1.5, to pH 5.5. This mild pH elution technique has no adverse effect on the fusion protein, either in its ability to bind to CEA or in its enzymatic activity (for methods, see Example J). Tab. 5 shows the OD values and fluorogenic units (FU) of the individual fractions from a purification using the solid phase-immobilized, anti-idiotypic MAb BW 2064/34.

Table 5:
Anti-idiotypic affinity chromatography

	Fractions	OD in %	FU in %	pH	Chromatography procedure
5	1-5	1	0	7.2	Preliminary washing of the column with PBS, pH 7.2
	6-142	20	0	7.2	Sample loading
	143-162	1	0	7.2	Washing of the column with PBS, pH 7.2
10	163	1	0	7.2	
	164	1	0	7.2	
	165	1	0	7.2	
	166	1	0	6.8	
15	167	2	10	6.1	
	168	5	20	5.7	
	169	16	40	5.6	
	170	23	80	5.5	
	171	26	100	5.4	Elution with PBS, pH 4.2
20	172	24	80	5.3	
	173	19	60	5.2	
	174	14	40	5.2	
	175	10	30	5.1	
	176	8	25	5.1	
25	177	6	20	5.1	
	178	3	10	5.0	
	179	2	5	5.0	
	180	1	0	5.0	

30 1 fraction = collection for 6.6 min (at pumping rate of 18 ml/h)
= 2 ml
The FU values are indicated as % of the highest value (fraction 171).

The lution of the fusion protein was measured as prot in
by measurement of the OD at 280 nm. In addition, the
isolated fractions were examined for specific binding to
CEA and simultaneous enzyme activity in the specificity/
5 enzyme activity assay (Example J). The values show that
all the specific binding and enzyme activity was con-
centrated in one peak (peak eluted from around pH 5.0 to
pH 5.6). The conclusion from this is that the described
method of anti-idiotyp affinity chromatography is a very
10 efficient and selective purification technique for the
431/26hum V_hhu β Glc 1H fusion protein.

Example (N):

Gel chromatography of the fusion proteins

The supernatants from the BHK cells secreting the
15 431/26hum V_h hu β Glc 1H fusion protein (B 70/6, B 74/2,
B 72/72, B 73/2) were removed, sterilized by filtration
and subjected to analytical gel filtration. For this, a
TSX G3000 SW-XL column (7.8 x 300 mm) was equilibrated
with 0.1 M sodium phosphate buffer, pH 5.7, + 0.02% NaN₃,
20 20 μ l of the supernatant were loaded on, and elution was
carried out with a flow rate of 0.6 ml/min. Starting with
an elution time of 9 min (exclusion volume 9.5 min),
fractions (0.3 min each) were collected and assayed for
 β -glucuronidase activity.

For this 25 μ l of the particular fraction were mixed with
75 μ l of substrate solution (2.5 mM 4-methylumbelliferyl
 β -glucuronide in 200 mM sodium acetate buffer, pH 5, +
0.1 mg/ml BSA) and incubated at 37°C for 2 hours. The
reaction was then stopped with 1.5 ml of 0.2 M
25 glycine/0.2% SDS solution, pH 11.7, and the fluorescent
label liberated by the glucuronidase was quantified in a
30 Hitachi fluorometer (with excitation wavelength of 360 nm
and emission wavelength of 450 nm).

Result:

5 All 4 constructs show a single main activity peak between fractions 4 and 6 (Table 6). This corresponds to retention times of about 10.2 - 10.8 min. The fusion proteins with glucuronidase activity in the supernatants thus have retention times which are of the same order of magnitude as those of chemically prepared antibody- β -glucuronidase constructs (10.4 min). The retention time for the free enzyme is 11.9, and for the free antibody is 12.3 min.

Table 6:

Gel filtration of various fusion proteins

Incubation: 25 μ l, 37°C, 120 min

Substance concentration: 1.875 mM; 0.1 mg/ml BSA

5 Each fraction 0.3 min; start at 9 min

		Liberated label/assay (FU)			
	Fractions	B70/6	B74/2	B72/72	B73/2
10	1	11	17	15	15
	2	22	35	44	38
	3	125	97	873	1014
	4	1072	196	2959	3994
	5	1588	165	2206	3141
15	6	1532	120	1133	1760
	7	941	103	581	926
	8	710	69	376	723
	9	500	108	302	626
	10	371	123	316	613
20	11	320	107	263	472
	12	254	91	210	456
	13	224	57	146	357
	14	190	65	134	332
	15	171	52	83	294
25	16	167	44	99	243
	17	100	38	73	217
	18	129	34	55	179
	19	75	45	48	155
	20	66	41	36	129
30	21				118
	22				93
	23				78
	24				61
	25				24
	26				51

Example (O):

Molecular characterization of the 431/26 hum V_H hu β Glc1H fusion protein

5 The fusion proteins were purified by anti-idiotypic
affinity chromatography in Example (M). Aliquots from the
peak eluted at pH 5.5 were subjected to 10% SDS PAGE
electrophoresis under non-reducing and reducing condi-
tions and immunostained in a Western blot using anti-
10 idiotypic MAbs or with anti- β -glucuronidase MAbs (Towbin
and Gordon (1979), Proc. Natl. Acad. Sci. USA 76: 4350-
4354).

Under non-reducing conditions with the 431/26 hum V_H
hu β Glc1H fusion protein, a main band of \approx 125 kDa and a
band of 250 kDa were detected and were detectable both by
15 anti-idiotypic MAb and by anti- β -glucuronidase MAb in the
Western blot. Under reducing conditions there was no
detectable immunostaining either by the anti-idiotypic or
by the anti- β -glucuronidase MAbs. A 100 kDa and a 25 kDa
band were detected in the reducing SDS PAGE. However,
20 these molecules analysed under denaturing conditions are,
according to TSK G 3000 SW-XL gel filtration under native
conditions in the form of a higher molecular weight
product which has a molecular weight of \approx 250 kDa
(Example N). Diagrammatic representations of the 431/26
25 hum V_H hu β Glc1H fusion protein are shown in Fig. M. Figure
Mb shows the monomer which has a \approx 25 kDa light chain and
a \approx 100 kDa heavy chain. This monomer and a dimer linked
by inter-heavy chain disulfide bridges can be detected
under denaturing conditions (Fig. Ma). Under native
30 conditions, the fusion protein is in the form of a dimer
of \approx 250 kDa, with or without inter-heavy chain disulfide
bridges (Fig. Mc).

EXAMPLE (P):**Chemical modification of the fusion protein**

The fusion protein purified as in Example (N) (110 $\mu\text{g/ml}$) was adjusted to pH 4.5 and mixed with sodium periodate (final concentration 1 mM). After incubation at room temperature in the dark for 1 hour, the sodium periodate was removed by gel chromatography, and the fusion protein was then readjusted to pH 8. Addition of ethanolamine to a final concentration of 0.1 M was followed by incubation at 4°C for a further 3 hours, then sodium cyanoborohydride (final concentration 5 mM) was added and incubated for 30 min (reduction). This was followed by another gel filtration to remove the reducing agent and to change the buffer of the fusion protein. The chemical modification had no effect on the functional activity of the fusion protein. Tab. 7 shows the change in the plasma concentrations of unmodified and modified fusion protein in the nude mouse. The elimination of the fusion protein from the plasma is greatly slowed down by the modification.

Table 7:

Plasma levels of β -glucuronidase activity in the nude mouse

25	t [min]	β -Glucuronidase fusion protein	
		Treated % activity	Untreated % activity
	0	100	100
	10		54
30	30	76	
	60		22
	240	40	4
	480		1
	1380	19	
35	1440		1
	5880	3	

EXAMPLE (Q):

Enzymatic treatment of the fusion protein

53 µg of fusion protein (Example N) in 0.01 M tris/HCl, 0.15 M NaCl were incubated with 1 unit of soluble alkaline phosphatase (E. coli) or immobilized alkaline phosphatase (bovine intestine) at room temperature for 20 h. Tab. 8 shows the change in the plasma concentration of untreated and treated fusion protein in the nude mouse. The elimination is not significantly affected by the enzyme treatment.

Table 8:

Plasma levels of β-glucuronidase activity in the nude mouse

		β-Glucuronidase fusion protein	
t	[min]	AP (bovine intestine, immobil.)	AP (E. coli)
		untreated [%]	treated [%]
20	0	100	100
	10	78	65
	30	44	53
	60	35	35
	240	22	22
25	1440	4	5

AP = alkaline phosphatase

Example (R):

Pharmacokinetics and tumor retention of the 431/26 hum V_h huβGlc 1H fusion protein

By way of example, 5 × 4 µg of purified fusion protein

which was mixed with 100 μ g of HSA/ml were injected in unmodified (Example N) and chemically modified form (Example P) i.v. at 24-hour intervals into CEA-expressing nude mice harboring human tumors. After defined time intervals, 3 animals in each case were sacrificed by cervical dislocation. The organs were removed, weighed and mixed with 2 ml of 1% strength BSA in PBS, pH 7.2. The tissue and cells from these organs were then broken down in a Potter (10 strokes) and the amount of functionally active fusion protein was determined in the supernatant after centrifugation of the suspension at 3000 rpm and RT for 10' (Heraeus Labofuge GL, Type 2202) in the specificity/enzyme activity assay (see Example J). The data from a representative experiment are shown in Tab. 9. It is clearly evident that the chemically modified fusion protein, which has a $t_{1/2\beta}$ of \approx 4 h, specifically accumulates in the tumor from \geq 3 days after completion of the repetitive injection phase. The unmodified fusion protein, which has a $t_{1/2\beta}$ of \approx 20 min, showed no significant accumulation in the tumor under the same experimental conditions.

It may be concluded from these data that the hu 431 β -Gluc fusion protein is able to bind in vivo to CEA-positive tumors and to remain there as enzymatically active molecule over long time periods ($>$ 9 days). The time the prodrug is administered in this system should be between day 3 and 9 after completion of the fusion protein injection.

Table 9:

Tumor retention experiment with CEA-positive human stomach carcinoma xenograft (Me Sto 1)

I.v. Injection of $5 \times 4 \mu\text{g}$ of fusion protein per mouse at intervals of 24 hours

Time (h) after last i.v. injection	% Activity/g of tumor or organ						
	Tumor	Liver	Lung	Spleen	Kidney	Intestine	Plasma
3	100	100	100	100	100	100	100
24	86.7	49.3	26.3	-	64	123	44.4
72	26.4	11	4.4	-	8.1	19	6.5
216	24.4	1.5	0.13	1.2	0.4	0.5	0.015

Example (S):

Isoelectric focusing of the 431/26 hum V_h hu β Glc 1H fusion protein

5 The fusion protein purified by anti-idiotypic affinity chromatography (Example N) was subjected to isoelectric focusing in the Pharmacia Fast System by the method of Righetti et al. (1979). It emerged from this that the isoelectric point of the molecule lies in a pH range from 7.35 to 8.15.

10 **Example (T):**

Demonstration that a cytostatic prodrug can be cleaved by the 431/26 hum V_h hu β Glc 1H fusion protein

15 It was shown in Example (J) that the β -glucuronidase portion of the fusion protein is able, like the native human enzyme, to cleave the β -glucuronide of 4-methylumbelliferol. In the investigations which are described hereinafter, the substrate used for the enzymatic cleavage was a β -glucuronide, linked via a spacer group, of the cytostatic daunomycin. The specific procedure for these investigations was as follows:

20 4 mg of the compound N-(4-hydroxy-3-nitrobenzyloxy-carbonyl)daunorubicin β -D-glucuronide (prodrug), which is described in French Patent Application (No. d'Enregistrement National: 9105326) were dissolved in 1 ml of 20 mM phosphate buffer, pH 7.2. 35 μ l of the fusion protein (Example N) or of human β -glucuronidase (total concentration in each case 6.5 U/ml; 1 U = cleavage of 1 μ mol of 4-methylumbelliferol/min at 37°C) were pipetted into 5 μ l portions of this substrate solution and incubated in the dark at 25 37°C. Samples (5 μ l) of the incubation mixture were removed after various times and immediately analyzed by high pressure liquid chromatography under the following conditions:

Column:

Nucleosil 100 RP 18, 5 μ m particle diameter, 125 x 4.6 mm

Mobile phase:

Gradient of solution A (100% acetonitrile) and solution B
(20 mM phosphate buffer pH 3.0)

0 min: 30% solution A

15 min: 70% solution A

20 min: 70% solution A

Flow rate: 1 ml/min

10 Detection: fluorescence, excitation 495 nm, emission 560 nm

Data analysis: Beckman System Gold Software

15 The retention time of the starting compound (prodrug) under these chromatography conditions was 11 min. The compound produced during the incubation (drug) had a retention time of 8.9 min, identical to daunomycin (DNM, analysis of a standard under the same conditions). The kinetics of the cleavage of the starting compound by the fusion protein and human β -glucuronidase are shown in Tab. 11 and Tab. 10 respectively.

20 The half-life of the cleavage of the prodrug by the fusion protein was 2.3 h. Cleavage by human β -glucuronidase took place with a half-life of 0.8 h. As already demonstrated in Example (J), the results of the investigations show that
25 the β -glucuronidase portion of the fusion protein is functionally active and able to cleave β -glucuronides. The kinetics of the elimination of the glucuronide portion and the liberation of the drug (daunomycin) from the prodrug used show a rate comparable in magnitude to human
30 β -glucuronidase, so that the substrate specificity of the fusion protein essentially agrees with that of human β -glucuronidase.

Table 10:

Kinetics of prodrug cleavage by β -glucuronidase (human, recombinant)

	t min	Prodrug area %	DNM area %
5	0	99.2	0.8
	57	36.0	64.0
	130	10.3	89.7
10	227	9.3	90.7

Table 11:

Kinetics of prodrug cleavage by β -glucuronidase (fusion protein)

	t min	Prodrug area %	DNM area %
15	0	98.9	1.1
	50	81.1	18.9
	135	51.7	48.3
20	190	33.0	67.0
	247	22.0	78.0
	317	12.4	87.6

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A fusion protein for prodrug activation of the formula
huTuMab-L- β -Gluc, where huTuMab is a humanized tumor-
specific monoclonal antibody or a tumor-binding fragment
5 thereof, L is a linker, and β -Gluc is human
 β -glucuronidase.
2. A fusion protein as claimed in claim 1, in which the
antibody fragment is composed of a V_H exon, a CH₁ exon
and a hinge exon and leads to expression of fusion
10 proteins which are not linked together by disulfide
bridges.
3. A fusion protein as claimed in claim 1, in which the
antibody fragment is composed of a V_H exon, a CH₁ exon
and two hinge exons and leads to the expression of
15 fusion proteins in which the heavy chains fragments
are linked together by disulfide bridges.
4. A fusion protein as claimed in claim 1, in which the
antibody fragment is composed of a V_H exon, a CH₁ exon
and three hinge exons and leads to the expression
20 of fusion proteins which are linked together by
disulfide bridges.
5. A fusion protein as claimed in claim 1, in which the
antibody fragment is composed of a V_H exon and a CH₁
exon and on expression can associate with a modified
25 light chain composed of V_L and CH₁ domain.
6. A fusion protein as claimed in any of claims 1 to 5,
where the huTuMab portion derives from MAb BW 431/36.
7. A fusion protein as claimed in any of claims 1 to 5,
where the huTuMab portion is formed from one of the
30 MABs described in EP-A1-0 388 914.
8. A fusion protein as claimed in any of claims 1 to 7,

in which L contains a polypeptide spacer as shown in Tab. 1 and/or 2.

- 5 9. A fusion protein as claimed in any of claims 1 to 8, in which L contains 1, 2 or 3 hinge regions of a human IgG3 C gene.
- 10 10. A plasmid which contains the cDNA for peptides as claimed in claim 1 to claim 9.
11. A transformed eukaryotic cell which is transformed with a plasmid as claimed in claim 10.
- 10 12. A process for the preparation of proteins as claimed in claim 1 to claim 9, which comprises expressing these fusion proteins in transformed cells by means of plasmids, and isolating said fusion proteins via anti-idiotypic MAbs.
- 15 13. A fusion protein as claimed in any of claims 1 to 9, which fusion protein is treated with an oxidizing agent.
14. A fusion protein as claimed in claim 13, which fusion protein is reductively aminated in a second reaction step.
- 20 15. A process for the preparation of fusion proteins as claimed in claim 13, which comprises treating the fusion proteins as claimed in any of claims 1 to 9 with an oxidizing agent.
- 25 16. A process for the preparation of fusion proteins as claimed in claim 14, which comprises the fusion proteins as claimed in any of claims 1 to 9 being oxidized in a first reaction step and reductively aminated in a second reaction step.
- 30 17. A fusion protein as claimed in any of claims 1 to 9 and claims 13 and 14 as pharmaceutical.

18. A fusion protein as claimed in any of claims 1 to 9 and claims 13 and 14 as diagnostic aid.

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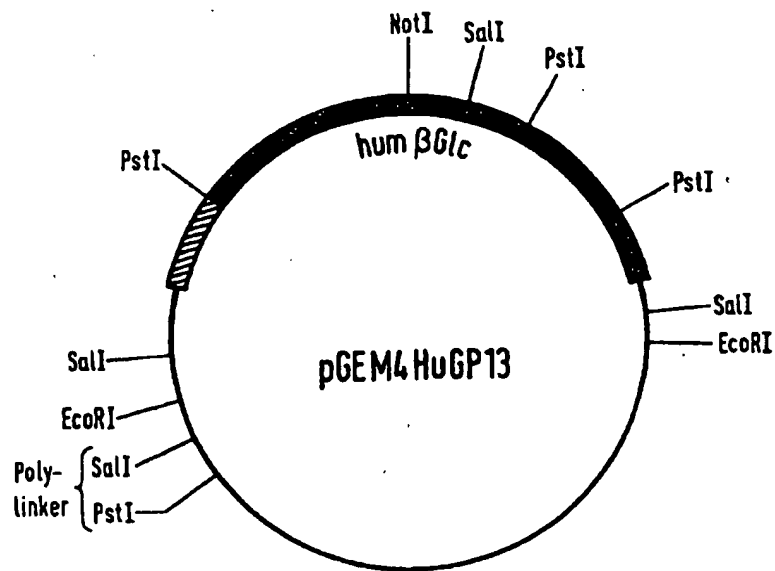


Fig. A

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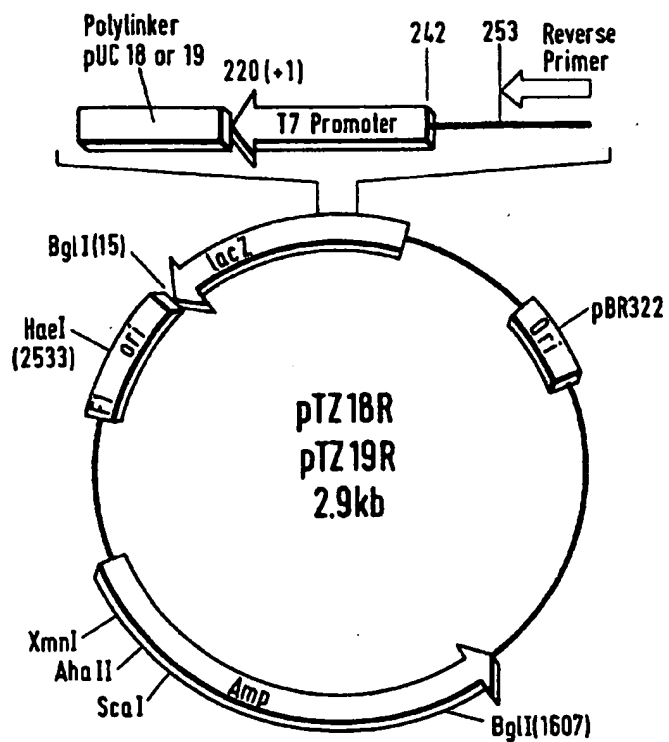


Fig. B.1

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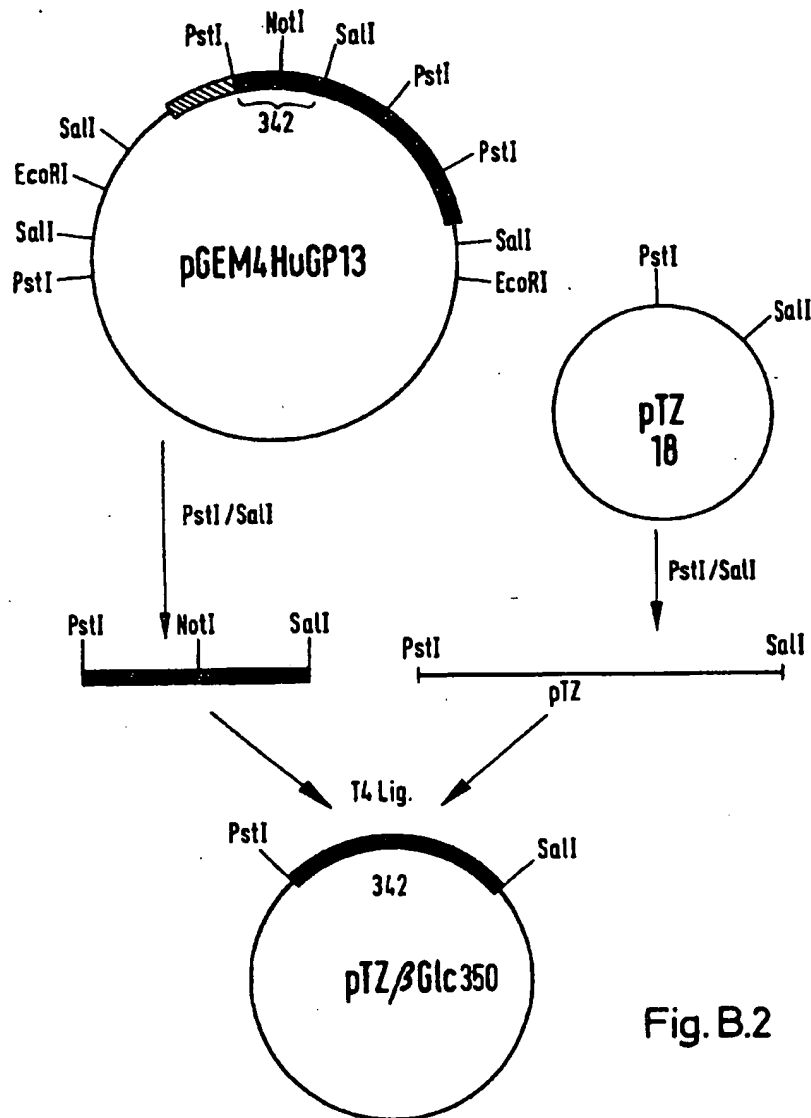
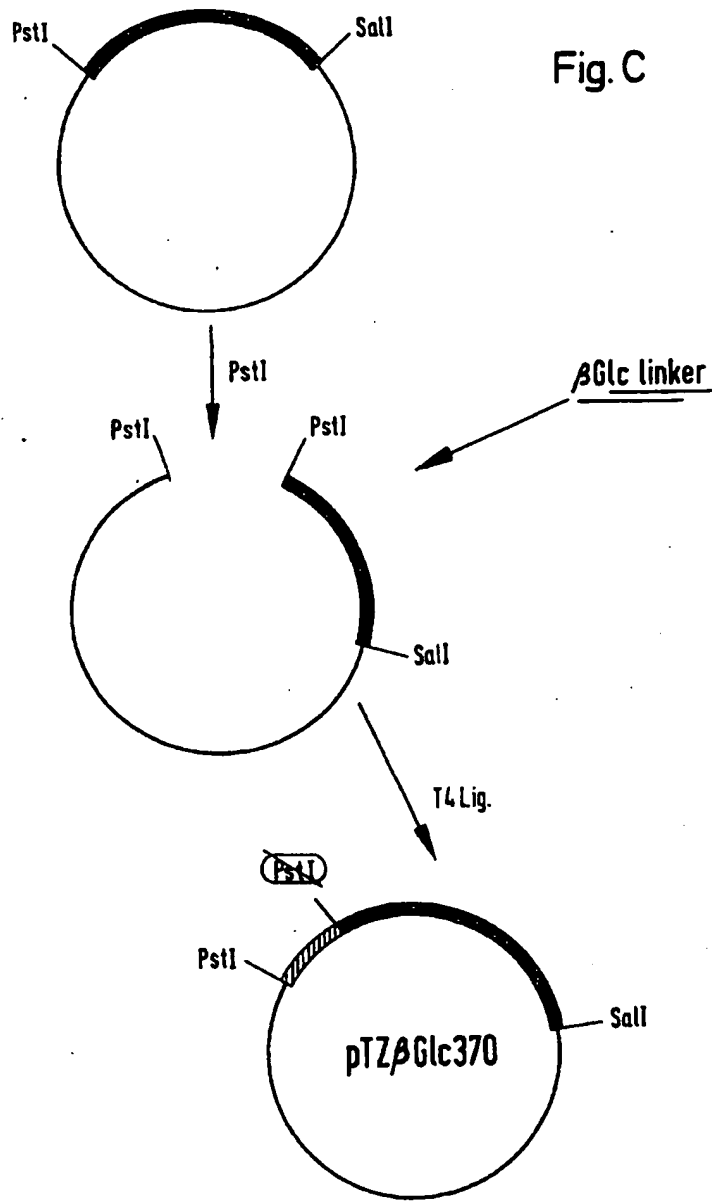


Fig. B.2

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Fig. C



By. Benoit & Pann

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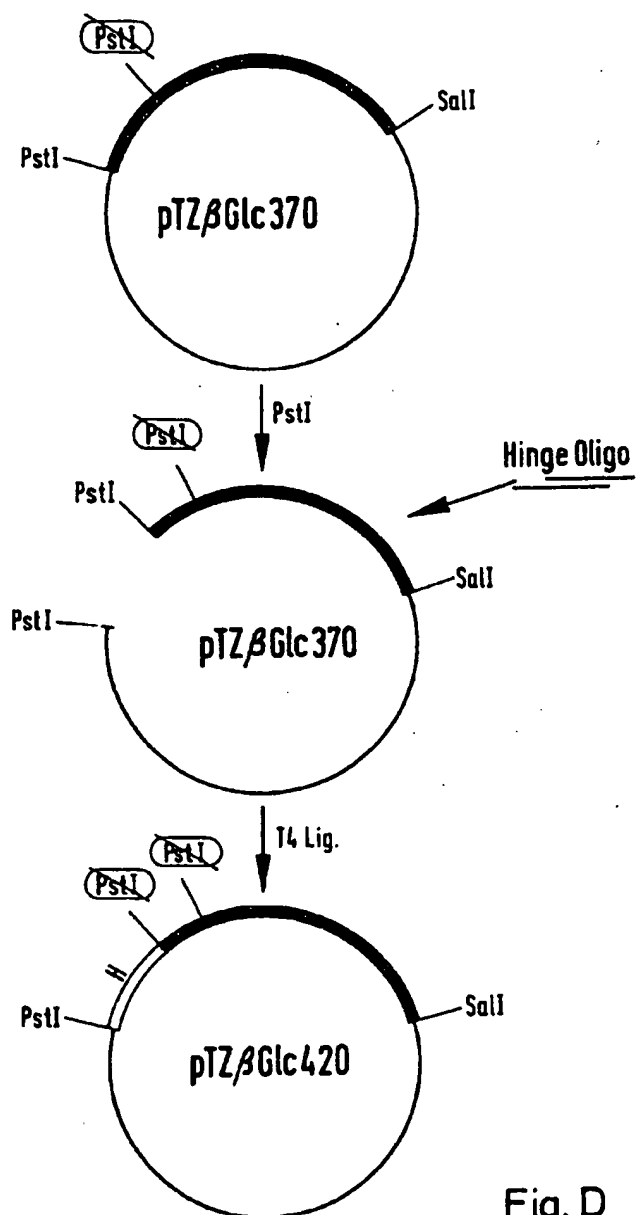


Fig. D

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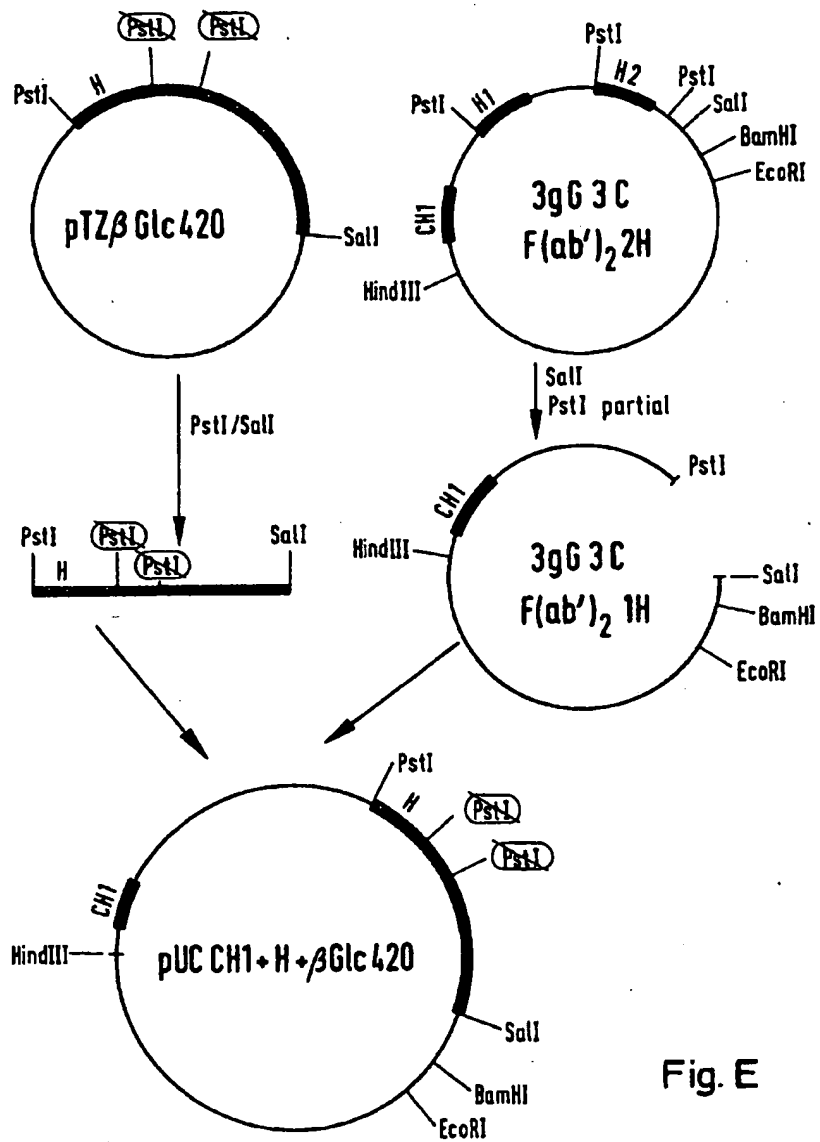


Fig. E

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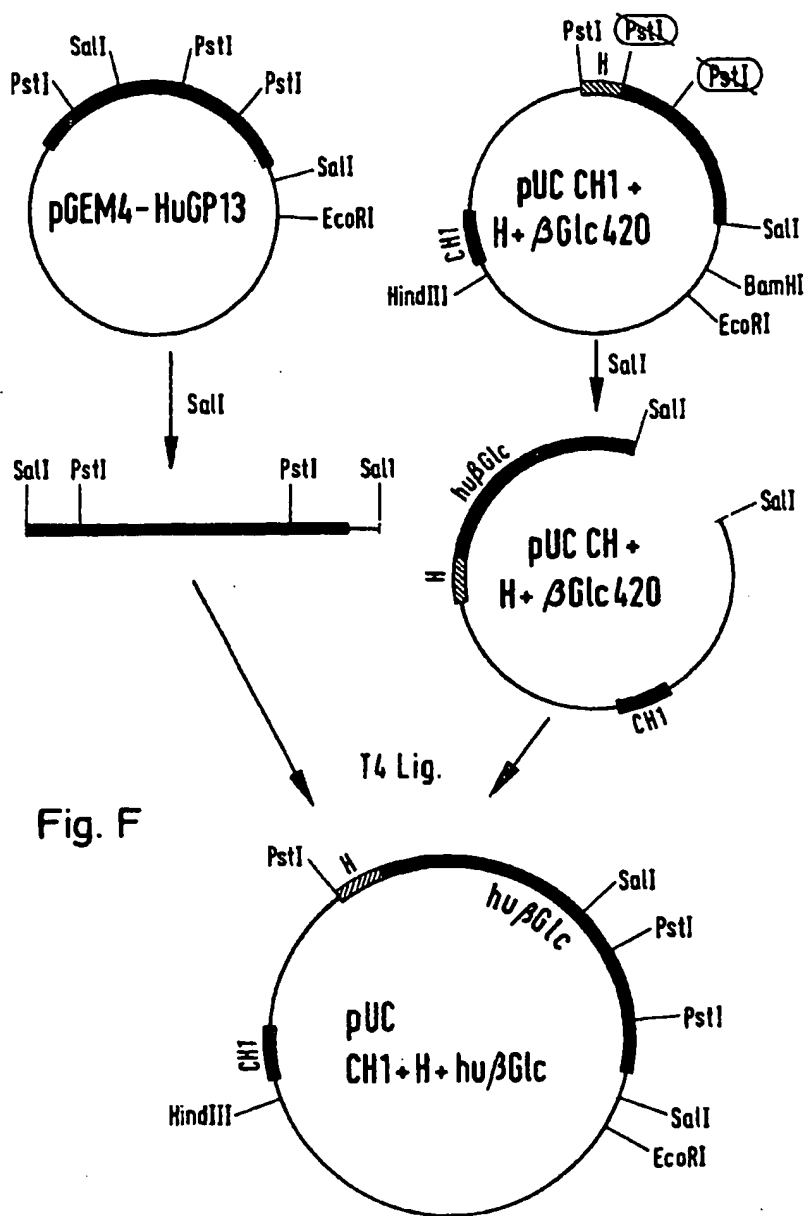


Fig. F

By: Gersbach & Pan

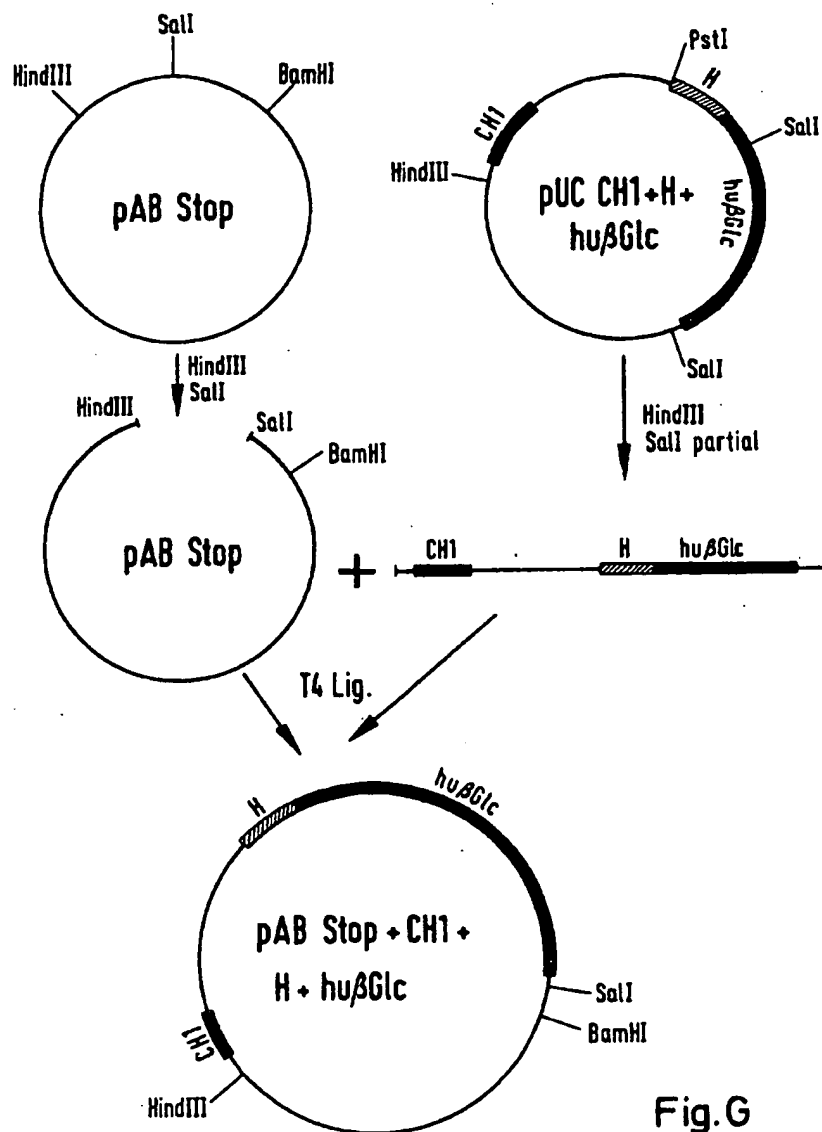


Fig.G

By: Breakey & Parr

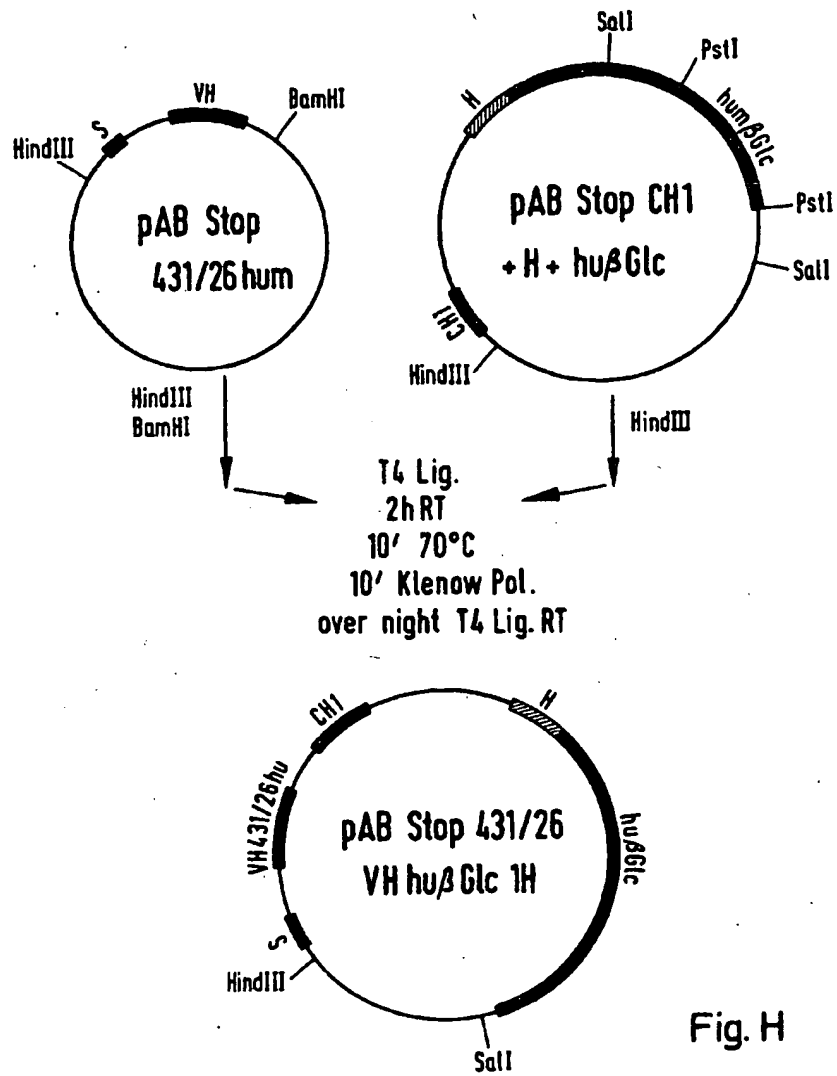


Fig. H

By: Genevieve & Pam

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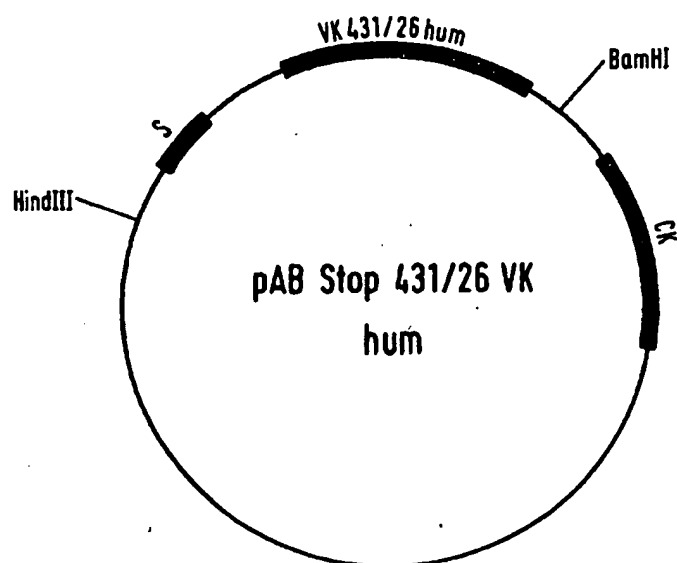


Fig. I

By. Bercovich + Parr

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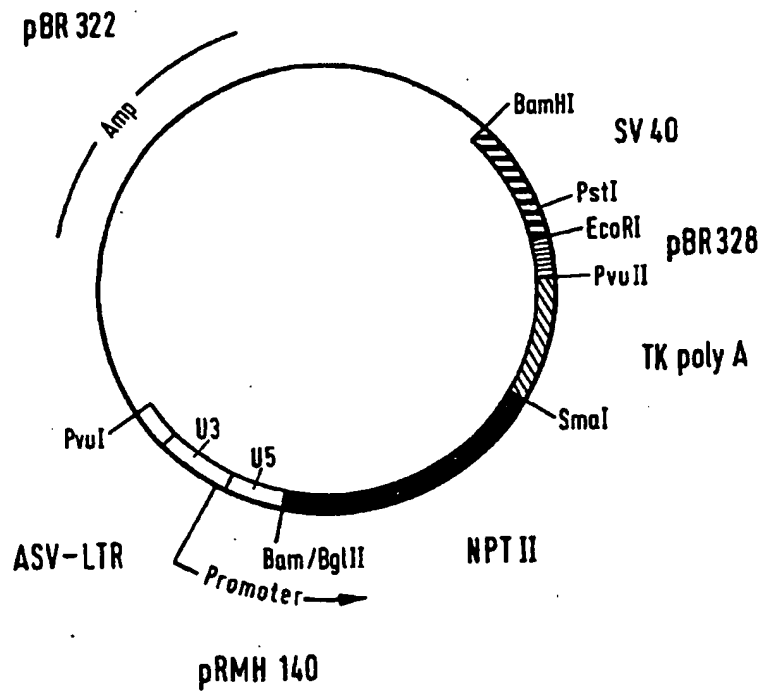


Fig. K

By: Bercovich + Pan

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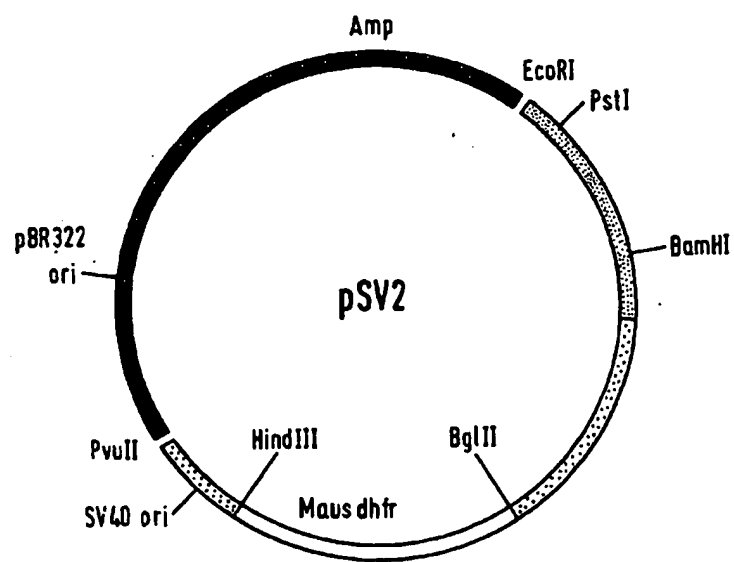
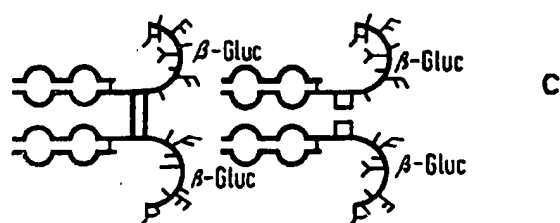
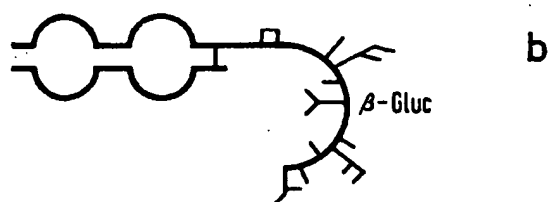
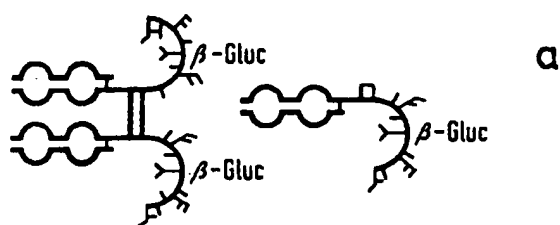


Fig. L

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Fig. M



by: Burstein & Pan

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